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# THE BLOOD CELLS AND TUNIC OF THE ASCIDIAN *HALOCYNTHIA AURANTIUM* (PALLAS). I. HEMATOLOGY, TUNIC MORPHOLOGY, AND PARTITION OF CELLS BETWEEN BLOOD AND TUNIC[1]

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The blood cells and tunics of a number of ascidians have been described (Seeliger and Hartmeyer, 1911; Hecht, 1918; St. Hilaire, 1931; George, 1939; Endean, 1955a, 1955b, 1960, 1961; Andrew, 1962; Freeman, 1964). There generally appears to be three categories of blood cells present: vacuolar, amoebocytic, and a lymphocytic stem type. High levels of transition metals have been reported in the blood cells of tunicates (Henze, 1911; Webb, 1939, and 1956; Bertrand, 1950; Endean, 1955a, 1955b, 1960, and 1961; Levine, 1962). Several functions have been ascribed to the blood cells of ascidians: tunic secretion (Ries, 1937; Endean, 1955b, and 1961; Kalk, 1963), phagocytosis, defense response to foreign bodies (Anderson, 1969) and trephocytosis (Liebman, 1947). It has been recognized for some time that the blood cells move into, and take up residence in, tunic material (Seeliger and Hartmeyer, 1911; Hecht, 1918; Endeon, 1955a, 1955b, 1960, and 1961). There is some disagreement concerning the source of the tunic, whether it is a blood cell secretion (Endean, 1955b, and 1961) or an epidermal secretion (Deck, Hay, and Revel, 1966). The morphology of the tunic of various ascidians is diverse (St. Hilaire, 1931) and this has complicated the problem of understanding the origin of tunic and blood cell function therein. There has been little quantitative consideration or statistical analysis concerning blood cell distribution and function.

The solitary ascidian, *Halocynthia aurantium* (Pallas), is commonly called the sea peach because of its coloration and general shape. It is considered to be the Pacific counterpart of the Atlantic species (*Halocynthia pyriformis* (Rathke) (Van Name, 1945). In Howe Sound, British Columbia, *H. aurantium* is seldom found at a depth of less than 10 meters and the animals are found in their greatest numbers between 10 and 20 meters; the greatest depth at which *H. aurantium* was encountered by the author was approximately 25 meters. At these depths the animal is not exposed by tidal fluctuations and it is below the brackish surface layers that occur in Howe Sound during Spring and Summer freshwater runoff. *H. aurantium* appears to prefer attachment to vertical rock faces exposed to the

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currents. *H. aurantium* is found in large populations which contain extensive size ranges of individuals, but these populations are distributed erratically through Howe Sound. In this species, the tunic is clean and free of detritus and settling or boring organisms. The gastropod mollusc, *Fusitriton oregonensis,* has been observed to prey upon *H. aurantium* both in the field and in the lab, but this is a rare occurrence and it is believed that *H. aurantium* is relatively free of predation (Smith, unpubl. obs.). *H. aurantium* accumulates iron with the greatest concentration per gram dry weight found in the blood cells and with the second highest concentration found in the tunic (Smith, 1969). This study was undertaken to determine the morphology of the blood cells and tunic of this hitherto uninvestigated ascidian. Statistical analyses of blood cell distributions, their partition in the tunic, and their reaction to experimental manipulation enable the formulation of tentative hypotheses concerning the relationships of certain blood cell types, the origin of the tunic, and the function of particular cell types in the tunic.

## Materials and Methods

The ascidian, *Halocynthia aurantium* (Pallas), was identified by the author based on the work of Van Name (1945). The ascidians were collected in the late summer and early fall of 1966 by scuba diving at 10 to 20 meters from the following locations in Howe Sound, British Columbia: Whytecliff Park, Copper Cove, Horseshoe Bay, Britania Beach, and Sunset Beach. Ascidians were held in the lab in constantly aerated sea water at 10° C. or 14° C for at least five days prior to experimentation. The ascidians were squeezed gently until sea water ceased to flow from the siphons, blotted dry, and weighed. Wet weights of whole animals were determined on a triple beam balance to 0.01 g. Dry weights to 0.1 mg were determined on a Mettler balance after the animals were dried for 48 hours at 110° C.

The following statistical methods were employed; analysis of variance, Keul's sequential test for mean differences, linear regression calculation and tests of the significance of calculated regressions, correlation coefficients (r), Student t-test, and t-test for mean differences in paired observations (Snedecor, 1956; Steel and Torrie, 1960; Li, 1964). Since per cent data tend to be binomial, all such data, which were to be used for tests of significance, were transformed to the angle equal to the arc sine square root of per cent (Li, 1964). F-ratios were considered significant if the probability that the event was a random occurrence was equal to or less than 0.05.

A small longitudinal incision was made in the subendostylar sinus area of the ascidian, a drop or two of blood was collected and mixed with a drop of 5% formalin in sea water on a clean microscope slide. The preparation was allowed to stand for one minute, smeared across the slide, and stained with Leishman's stain in methanol. After staining, the smear was very rapidly dehydrated through two changes of 100% tertiary butanol, one change of 50% t-butanol 50% xylene, cleared through two changes of xylene, and mounted in "Permount." Slides prepared in this manner display good stain differentiation, but the staining quality deteriorates within several weeks.

For fixation of tissues or whole animals, buffered formalin with cetyl pyridinium

bromide in distilled water (Culling, 1963) was used. Other fixatives employed were 5% formalin in sea water, Bouin's, Baker's, and Carnoy's (Culling, 1963). Whole animals or tissues were fixed for 4 hours in Carnoy's and 24 hours in all other fixatives. After fixation, tissues were washed, dehydrated in graded ethanols, cleared in benzene, and embedded in Paraplast (M.P. 55.6° C.). Trimmed blocks were cut at 8 microns on a Spencer microtome. Frozen sections were cut from material, which had been fixed for 12 hours in cetyl pyridinium formalin, at 10 to 16 microns on an International cryostat.

Although several hematoxylin stains were attempted, the chrome hematoxylin with phloxine method of Gomori (1941) was found to be most satisfactory. An alternative stain for tunic material was aldehyde fuchsin with light green (Pearse, 1961). For the histochemical determination of iron, Perl's and Turnbull's methods for ferric iron and the dinitroresorcinol method of Humphrey were employed (Pearse, 1961). Some sections to be stained for iron were treated with 30% hydrogen peroxide and 5% oxalic acid to unmask protein bound iron (Dales, 1965). Osmium fixed blood cells were prepared by exposing fresh blood smears to osmium tetroxide vapors in a sealed coplin jar for one hour after which the smears were dehydrated, cleared, and mounted.

Differential cell counts were made from duplicate smears of blood from wet weighed animals. 100 cells per slide or 200 cells per ascidian were counted. Mean differential distributions of blood cells were obtained for 80 animals over a weight range from less than 1 to 80 grams. The group of 80 animals was broken into four sub-groups of twenty gram weight range each containing 20 animals. Per cent distribution values were transformed by the arc sine method, and the distribution of particular cell types was tested by the analysis of variance for differences as a function of weight group. If a significant F ratio was obtained for any cell type, the weight group means for that cell type were tested for mean differences by Keul's method.

Hematocrits were measured in Kolmer centrifuge tubes after 10 minutes centrifugation at 3000 rpm on an Orbit centrifuge. Total number of cells per cubic millimeter of blood was determined for 35 animals as a function of wet weight group by duplicate hemacytometer counts. Blood was diluted with 5% formalin in sea water which was 0.1% in eosin. Plasma pH was determined directly with a Beckman research model pH meter at room temperature.

Tunic was injured by extirpating a 5 by 10 mm section of tunic without damaging the epidermis. Samples with damaged epidermis were not used for cell counts. At 0, 1, 3, 5, 10, and 15 days after injury, six ascidians were fixed and the injured area was removed for sectioning. Sections of tunic and epidermis were cut at right angles to the long axis of the ascidian. All the cells in the tunic, from the epidermis to the exterior were counted as a function of their position relative to the epidermis in terms of numbers of cells per ocular field from the epidermis. The distance from epidermis to exterior was measured with an ocular micrometer, the field diameter was known, and the width of section (8 microns) was known. Consequently, cell counts could be expressed as number of cells per $mm^3$. of tunic and were comparable with blood concentration. Stolons, finger-like villi that form the hold-fast, were sectioned at right angles to their long axis. Cell counts in the tunic of stolons grown in the laboratory were made at the half dis-

tance from the growing tip of the stolon to the body wall of the animal. The morphology of body wall tunic and the tunic of natural and laboratory grown stolon was examined in serial cross section and representative longitudinal sections. All tunic material used for comparisons of cell concentration was from animals weighing 20 to 40 grams and was compared with blood values of that weight range.

A suspension of Aqua-dag colloidal carbon, 5% solids in sea water, was injected into the tunic of the right lateral area of a group of 20 to 40 gram ascidians. At 0, 3, 6, 12, 24, and 48 hours subsequent to injection, blood was drawn from the subendostylar sinus of six animals per time period and fresh and Leishman's stained smears were prepared. For each animal two smears were prepared and approximately 200 cells per animal were counted. The per cent and type of cell with carbon particles was determined. Per cent data were transformed by the arc sine method and analysis of variance and mean comparisons as a function of time after injection were calculated.

## Results

### *I. Wet* vs *dry weight*

There is a linear relationship between wet and dry weights of *H. aurantium*. The calculated correlation coefficient (r) is +0.997 ($n = 29$). Regression analysis reveals a slope value of 0.065. The probability that the calculated slope is due to random variability is less than 0.001.

### *II. Blood cell morphology*

*A. Mature morula cells* (Fig. 1A) comprise 2.9% of the blood cells based on a sample of 80 animals. They are between 9 and 10 microns in diameter with an anucleolar nucleus. The mature morula cell is filled with 1 to 2 micron vacuoles. The number of these vacuoles varies, but is seldom less than twenty. Under bright field illumination this cell is highly refractive and light green in color. The contents of the vacuoles are homogeneous under both phase and bright field illumination and acidophilic to Leishman's stain. Osmium vapors cause a light brown to gray coloration of the cell. The mature morula cell is distinguished from other vacuolar cells by the small size and large number of acidophilic vacuoles, all of which are smaller in diameter than the nucleus.

*B. Immature morula cells* (Fig. 1B), which are approximately 9 microns in diameter, comprise 28.1% of the blood cells. The cell is filled with 5 to 15 vacuoles which are each approximately equal to the nucleus in diameter (3 microns). Staining reactions are similar to the mature morula cells: vacuoles are acidophilic, green in live cells, and turn gray after exposure to osmium vapors. The number of vacuoles is lower than in mature morula cells and the vacuoles are consistently larger. Transition stages between the immature and mature morula cells are considered immature morula cells if any of the vacuoles present are equal to or larger than the nucleus in diameter and are five or more in number.

*C. Compartment cells* (Fig. 1C) make up 1.0% of the blood cells, are about 8 microns in diameter, and have 2 to 4 vacuoles which are larger than the nucleus. The contents of the vacuoles are homogenous and staining reactions are similar to

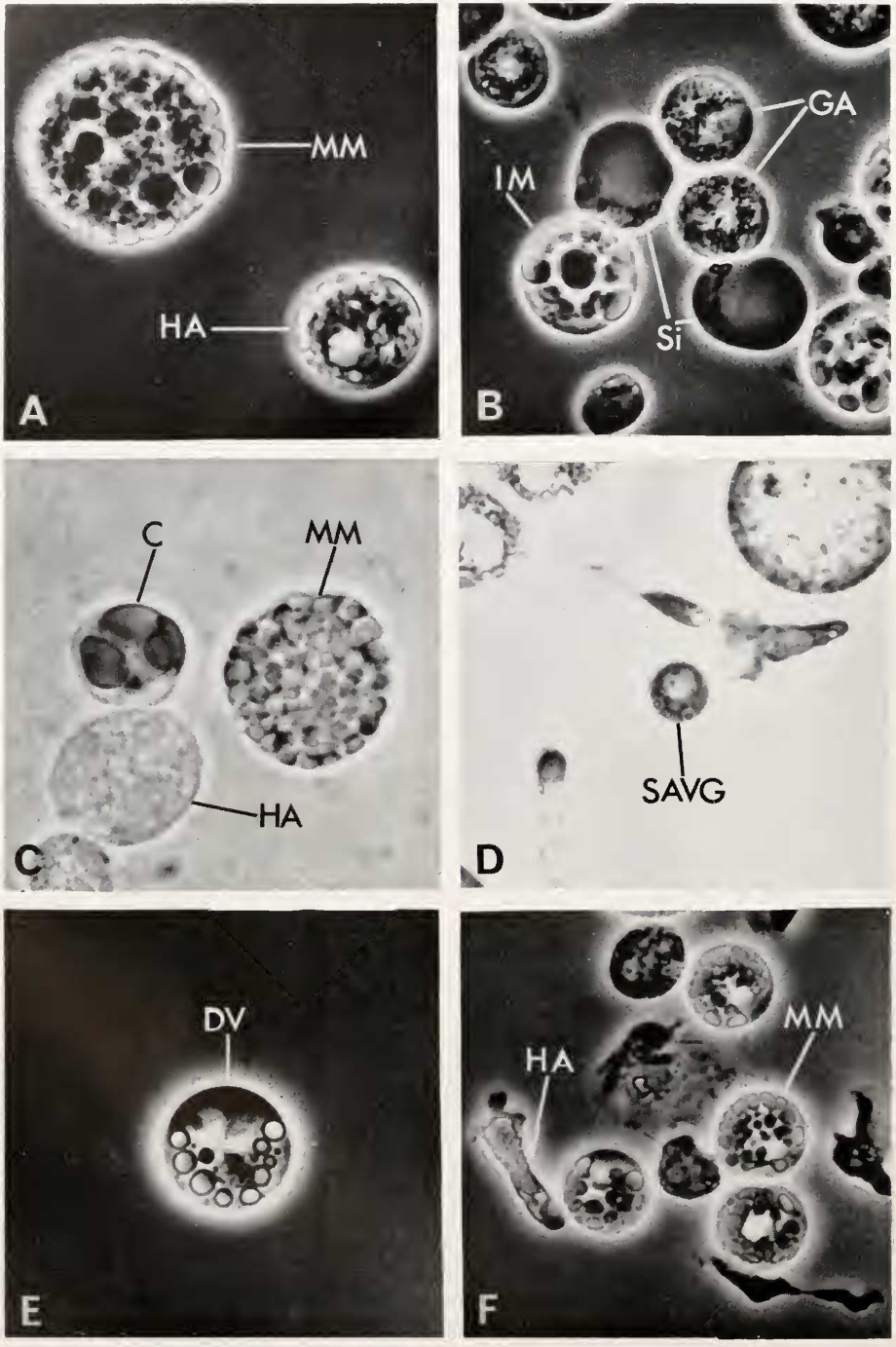


FIGURE 1. The blood cells of *H. aurantium*. Plates A (3000 ×), B (3000 ×), D 2000 ×), E (2000 ×), and F (1250 ×) are photographs of live cells taken through phase optics. Plate C (2000 ×) is a photograph of Leishman stained smear taken through bright field optics. The following notation is used: MM, mature morula cell; IM, immature morula cell; GA, granular amoebocyte; Si, signet ring cell; HA, hyaline amoebocyte; C, compartment cell; SAVG, stem cell with acidophilic vacuoles or granules; and DV, dispersed vesicular cell.

the immature morula cell. No nucleoli were observed in this cell type. These cells are distinguished from the immature morula cell by the larger size and lower number of vacuoles.

*D. Signet ring cells* (Fig. 1B) have one vacuole which fills the cytoplasm, pressing the nucleus to one side. They comprise 1.7% of the cells in circulation and are on the order of 6 microns in diameter. A cell is considered a signet ring when the vacuole is larger than the nucleus and when the vacuole does not contain granular inclusions. The vacuole is acidophilic or, more rarely, neutrophilic. The nucleus may occasionally contain a small nucleolus.

E. *Hyaline amoebocytes* (Fig. 1A, C, F) comprise 38.5% of the blood cells and range from 6 to 12 microns in diameter. The nucleus occasionally has a nucleolus. The cytoplasm is homogeneously neutrophilic to Leishman's stain. The cell may contain one or more vacuoles which enclose a small granular inclusion that is subject to Brownian motion in live cells. The cells may contain a large yellow grain. Under phase illumination, the cytoplasm appears reticulated or clumped. These cells are extremely amoebocytic and assume a variety of bizarre forms when viewed in live smears.

F. *Granular amoebocytes* (Fig. 1B) make up 20.5% of the cells in circulation. They have a discrete size range of 6 to 8 microns and are packed with distinct granules approximately 0.2 microns in diameter. The granules have some refractive properties when viewed under bright field illumination. Leishman's stained smears and phase contrast studies show that the granules do not fill the cytoplasm but leave a peripheral hyaline area. The granules are neutrophilic. Under phase illumination, the granules are seen to be associated with a reticular network.

G. *Stem cells* are 5 to 6 microns in diameter and make up 4.8% of the blood cells. The nucleus, which almost fills the cytoplasm, contains one or two small nucleoli. The chromatin is dispersed and the cytoplasm stains slightly basophilic with Leishman's stain. Under bright field and phase illumination, the cytoplasm of live cells appears hyaline and the nucleus looks clear except for a dark nucleolus.

H. *Stem cells* with acidophilic vacuoles or granules (Fig. 1D) are essentially similar to the stem cell except that they may be larger (5 to 7 microns), have a smaller nucleus with nucleolus, and have vacuoles and/or granules which are acidophilic to Leishman's stain. Vacuoles, when present, are always smaller than the nucleus.

I. *Giant stem cells* are rarely observed in the blood, and comprise only 0.2% of the cells. They are quite large (9 to 10 microns in diameter) with a large nucleus (5 microns) and a large nucleolus. The staining reactions of the giant stem cells are essentially similar to the stem type. Live giant stem cells are actively amoeboid and their cytoplasm is generally homogeneous, although occasionally it displays a fine granulation.

J. *Dispersed vesicular cells* (Fig. 1E) are of a uniform size (8 microns) and the cytoplasm contains 8 to 12 discrete vesicles of approximately 1 micron in diameter which are regularly dispersed throughout the cytoplasm. The vesicles are difficult to observe in live cells under bright field illumination, but under phase contrast they appear light against a dark background. With Leishman's stain, the cytoplasm is faintly basophilic and the vesicles faintly acidophilic. With

Gomori's chrome hematoxylin, the vesicles stain an intense blue-black. If the oxidation step of the procedure is omitted, the vesicles stain with phloxin. The nucleus often has a nucleolus.

None of the blood cells in *H. aurantium* stain for the presence of iron unless the sections are treated with hydrogen peroxide and oxalic acid. Subsequent to such treatment, the signet ring, the compartment, immature morula, and mature morula cells stain for iron. The staining intensity varies both within cell types and between cell types. This is probably due to a loss of the metal as it is unmasked by the action of the reagents.

### *III. Hematology*

The mean number of cells per $mm^3$ of blood from *H. aurantium* is 17,240 (n = 35, S.E. = 1690). Analysis of variance for mean number of cells per $mm^3$ as a function of 20 gram wet weight group displays an insignificant F ratio. The mean hematocrit is 0.38% (n = 15, S.E. = 0.06%). The mean corpuscular volume is 220 cubic microns. Mean plasma pH is 7.31 (n = 6) with a range from 7.20 to 7.41.

The mean differential distribution of blood cell types, derived from the 80 animal sample, is given with the description of the cell types (see above). The analyses of variance of per cent distribution of particular cell types as a function of 20 gram wet weight group gives significant F ratios in the following categories of cells: mature morula, stem, stem with acidophilic vacuoles or granules, dispersed vesicular cells, and hyaline amoebocytes (Table I). The compartment cells, immature morula, and signet ring cells do not show significant F ratios as a function of weight group when considered as individual types. If these cells, however, are grouped together into a category of *vacuolar cells,* the analysis of variance gives a significant F ratio as a function of weight group (Table I). The stem, stem with acidophilic vacuoles or granules, dispersed vesicular cells, mature morula, and *vacuolar cells* decrease with increased weight of animal (Fig. 2). The hyaline amoebocytes increase with increased weight of animal (Fig. 3). The 20–40,

TABLE I

*The relative concentration of blood cell types in the blood of* H. aurantium *as the mean per cent of particular cell type for each twenty gram wet weight group. The F ratio and P level from analyses of variance of per cent distribution of particular cell types as a function of wet weight group is given*

| Cell type | Mean per cent* | | | | F | *P* |
|---|---|---|---|---|---|---|
| | 0–20 | 20–40 | 40–60 | 60–80 | | |
| Mature morula | 4.8 | 2.5 | 2.4 | 1.7 | 6.188 | <0.005 |
| Dispersed vesicular | 2.6 | 1.9 | 1.2 | 0.9 | 3.969 | <0.025 |
| Vacuolar | 35.2 | 31.7 | 27.0 | 27.4 | 2.789 | <0.05 |
| Stem | 6.7 | 4.7 | 4.7 | 2.9 | 3.752 | <0.025 |
| S.A.V.G. | 1.8 | 0.9 | 0.9 | 0.5 | 7.173 | <0.005 |
| Hyaline amoebocyte | 27.7 | 37.3 | 45.1 | 45.1 | 13.286 | <0.025 |

*Mean per cent of cell type in the indicated weight range (g).

40–60, and 60–80 gram weight groups have significantly higher numbers of hyaline amoebocytes than the 0–20 gram group. The levels of significance of difference of mean per cent, after transformation by the arc sine method, of stem cells, stem cells with acidophilic vacuoles or granules, dispersed vesicular cells, mature morula, and vacuolar cells are given in Table II.

*IV. The Epidermis*

The epidermis (Fig. 4B) is a columnar epithelium with basophilic cytoplasm. The nuclear chromatin is diffuse and a nucleolus is rarely observed. The cells measure approximately 6 by 8 microns and frontal sections show that they are

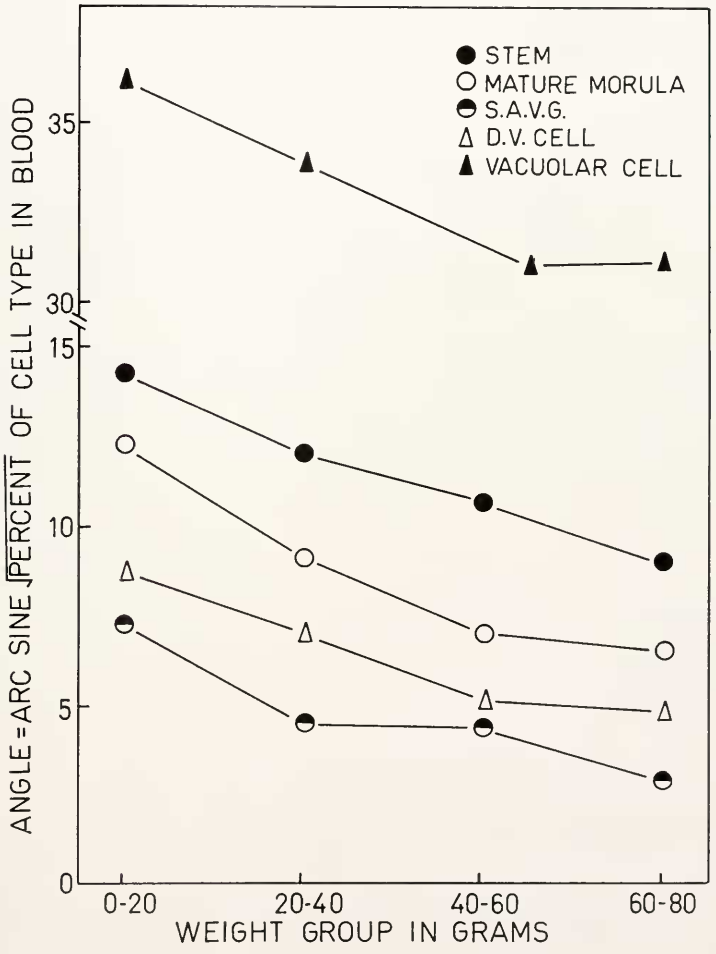


FIGURE 2. The per cent distribution of blood cell types in blood as a function of 20 gram whole animal wet weight groups of *H. aurantium*. Each point is the mean value for twenty animals in that weight group. *Vacuolar* cells refers to a collective group including the immature morula, compartment, and signet ring cell types.

TABLE II

*The significance of differences (Keul's method) of mean per cent of various blood cells as a function of 20 gram wet weight group in* H. aurantium

| Weight range | Cell type | PX Difference | | |
|---|---|---|---|---|
| | | −(60–80) | −(40–60) | −(20–40) |
| 0–20 grams | Stem | 0.01 | 0.01 | 0.01 |
| | S.A.V.G. | 0.01 | 0.01 | 0.01 |
| | *Vacuolar* | 0.01 | 0.01 | 0.01 |
| | M.M. | 0.01 | 0.01 | 0.01 |
| | D.V.C. | 0.01 | 0.01 | 0.01 |
| 20–40 grams | Stem | 0.01 | 0.01 | |
| | S.A.V.G. | 0.01 | N.S. | |
| | *Vacuolar* | 0.01 | 0.01 | |
| | M.M. | 0.01 | 0.05 | |
| | D.V.C. | 0.01 | 0.01 | |
| 40–60 grams | Stem | 0.01 | | |
| | S.A.V.G. | 0.01 | | |
| | *Vacuolar* | N.S. | | |
| | M.M | 0.01 | | |
| | D.V.C. | N.S. | | |

polygonal; each cell is generally surrounded by six other cells. No pores, channels, or vessels extend through the epidermis of the body wall into the tunic. Fibers of tunic material protrude from the apical ends of the epidermal cells. The epidermis is attached to a basement membrane from which connective tissue sheets extend through the subepidermal blood space to the circular and longitudinal muscles of the body wall.

### *V. Tunic*

The thickness of the tunic removed from fixed animals displays great variation, ranging from 0.60 mm for a 1.5 gram animal, to 1.20 mm for a 60 gram ascidian.

The greatest portion of the tunic is made up of a fibrous matrix (Fig. 4A) in which blood cells may be observed. The fibers, which extrude from the apical ends of the epidermal cells, coalesce just distal to the epidermis to form a fibrous layer or lamina. The matrix of tunic consists of a regular arrangement of these laminae, which form successive sheets of tunic material around the animal. The laminae are joined together by fibrous extensions between them. In larger animals, the laminae are grouped into bands; within a band, laminae are approximately of the same thickness, but the dimensions of laminae between bands differ. Cross sections of tunic from larger animals show bands of thick and thin laminae. There is a positive correlation between the number of laminae and wet weight of animal, $(r) = +0.95$ $(n = 28)$. Linear regression analysis of the number of laminae as a function of wet weight gives a slope of 1.67 and an intercept of 25.5. Confidence limits at the 0.95 level for the estimation of number of laminae from a given weight have been calculated (Fig. 5).

The external surface of the tunic is formed of papillar outgrowths of the matrix, upon which are situated cuticular spinous processes (Fig. 4C). Towards the periphery of the tunic the laminar structure is lost gradually and the fibers extend outwards forming a papilla. The mean height of papilla and cuticular spine is 271 microns ($n = 11$, S.E. $= 28$ microns). The spine is approximately 160 microns ($n = 11$, S.E. $= 7$ microns) in width at its base. The spine tapers from its base to an extremely fine point. The fibers of the matrix pass up

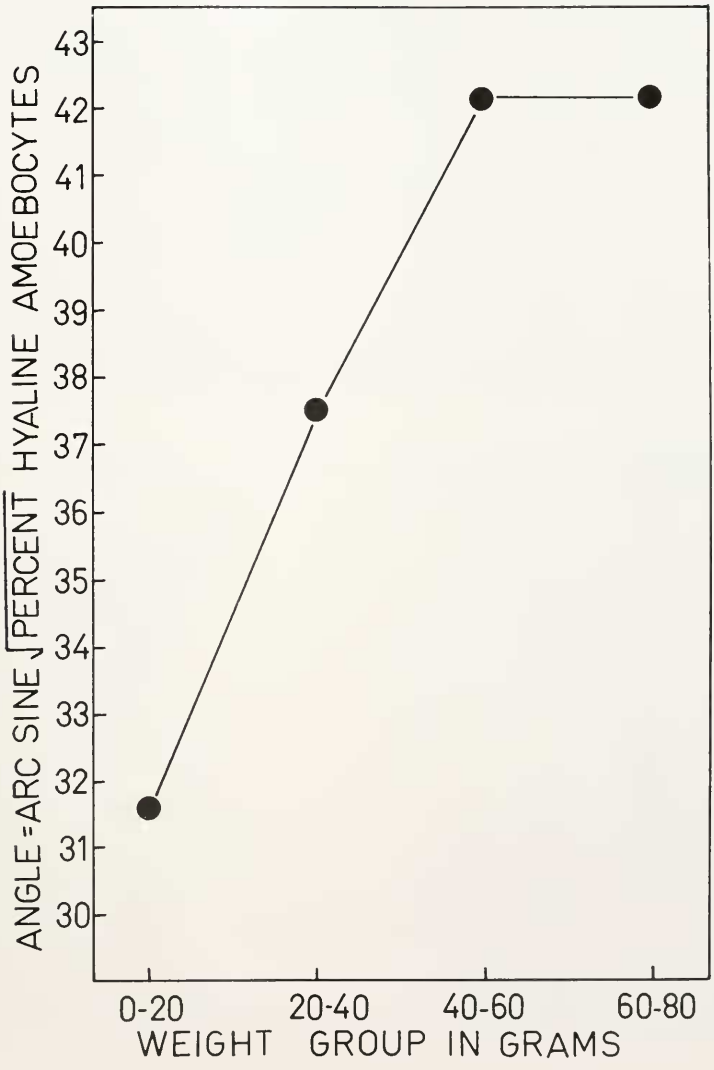


FIGURE 3. The per cent distribution of hyaline amoebocytes in the blood of *H. aurantium* as a function of 20 gram whole animal wet weight group. Each point is the mean value determined for 20 animals in that weight group.

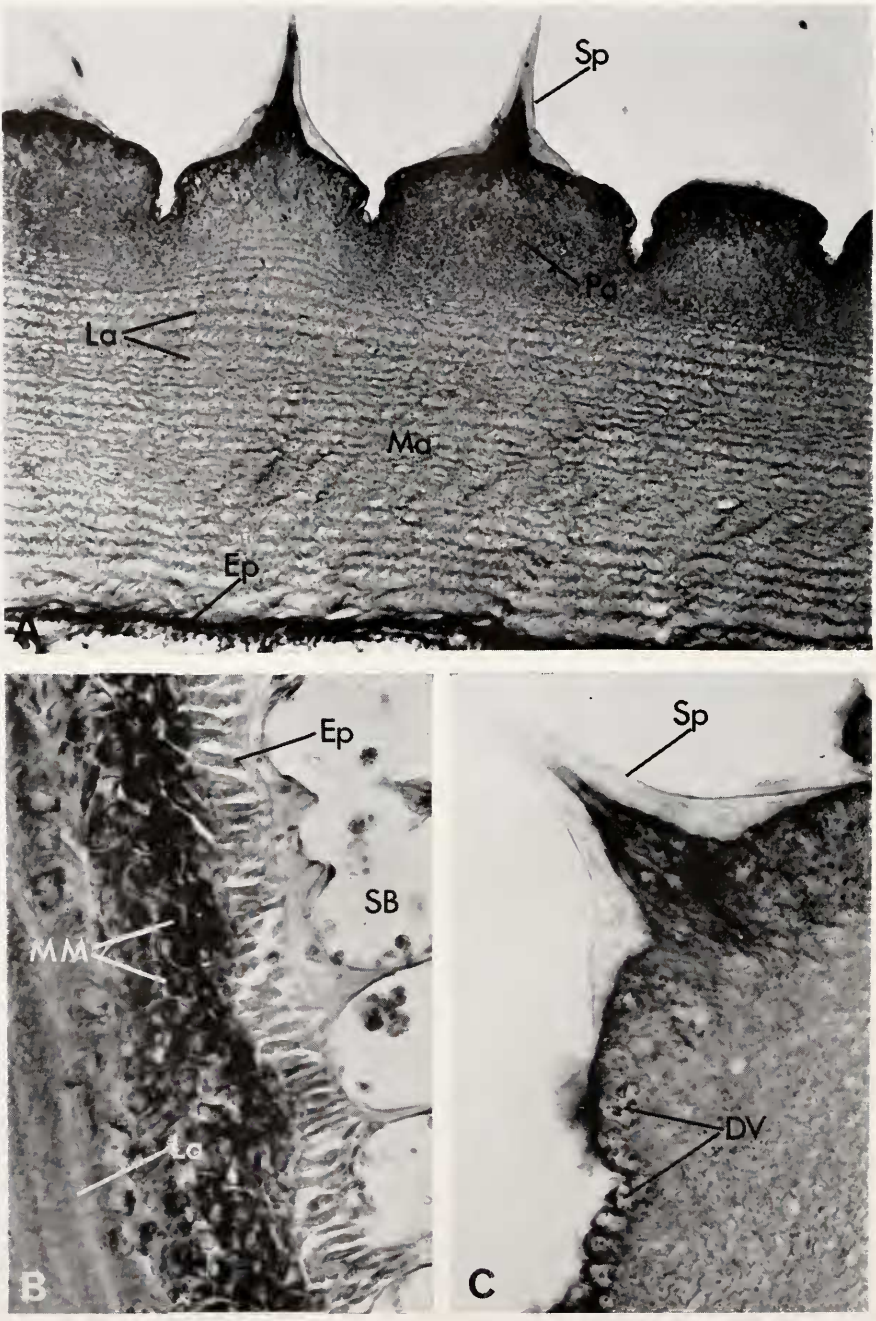


FIGURE 4. The tunic of *H. aurantium* stained with Gomori's hematoxylin and phloxine. Plate A (120 ×) is a cross section of body wall tunic. Plate B (650 ×) is a cross section at the level of the epidermis with an aggregation of mature morula cells just above the epidermis. Plate C (400 ×) is a cross section of a papilla bearing a tunic spine. The following notation is used: Sp, tunic spine; La, laminae; Ma, matrix; Ep, epidermis; SB, subepidermal blood space; MM, mature morula cell; and DV, dispersed vesicular cell.

the spine for some distance. The spine has a hollow core which passes all the way to the tip, and often contains blood cells. The material of the spine is homogeneous. Between the papillae, the surface of the matrix is overlaid with a thin layer of dense staining cuticle.

*VI. The stolons*

The hold-fast of *H. aurantium* consists of a number of stolons (Fig. 6A) which are finger-like outgrowths of the tunic, the underlying epidermis, and subepidermal blood spaces. The epidermis of stolons is more cuboidal than that of

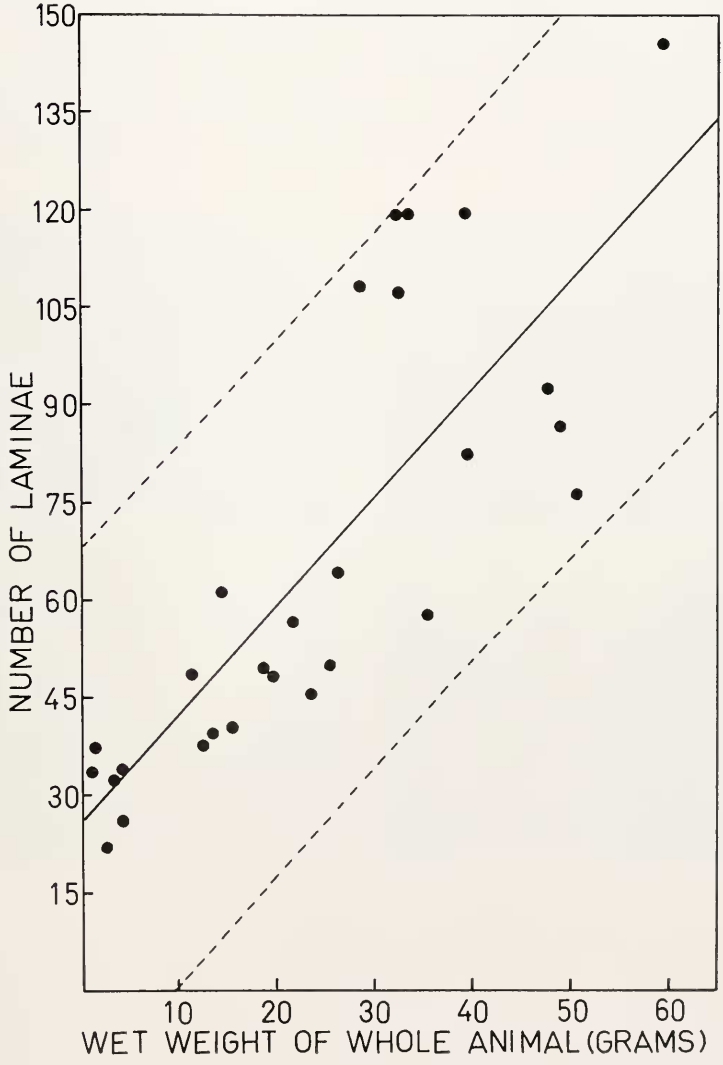


FIGURE 5. The number of laminae, or fibrous layers, in the tunic as a function of whole animal wet weight in grams. The solid line represents the calculated regression line. The dashed line represents the 95% confidence limits in the estimation of a $y$ value for any given $x$ value. The correlation coefficient is +0.95.

the body wall. The tunic matrix material of natural hold-fast stolon is irregular and tunic papillae and spines are absent although a dense staining cuticular surface is evident. In the laboratory, stolon material grows freely and rapidly, circumscribing the base of the ascidian. Over several months, these stolons will grow to lengths of approximately 1 mm and to diameters of 0.1 to 0.2 mm. The tunic matrix fibers of laboratory stolon show more regularity of structure than does the natural stolon matrix. At the distal tip of the laboratory grown stolon, the matrix material is quite thin and irregular (Fig. 6A). Proceeding from this tip along the stolon to the body of the animal there is an increase in the thickness of the matrix and an increase in the regularity of fibrous structure, until the matrix assumes a laminar appearance like that found in body wall tunic. The growing tip of laboratory stolon is covered with a thin cuticle, but near the body of the animal, papillae and cuticular spines are evident. There is a gradation of development of matrix, papillae, and cuticular spines from the distal tips of stolons to its juncture with the body wall.

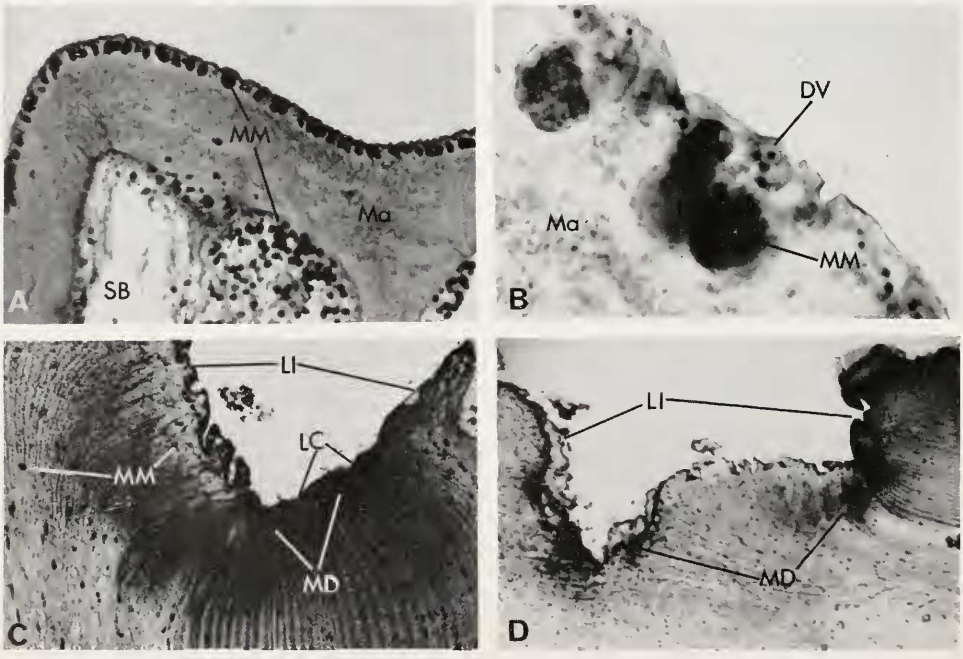


FIGURE 6. Laboratory grown stolon and injured body wall tunic of *H. aurantium* stained with Gomori's hematoxylin and phloxine. Plates A (200×) and B (1000×) are cross sections of stolon near the growing tip; note the aggregation of mature morula cells (MM) and dispersing vesicular cells (DV) peripherally and the lack of lamination of the tunic matrix (Ma). Plates C (100×) and D (50×) are cross sections of tunic five days after injury. In plate D the epidermis is to the bottom; note the dark staining mature morula cell degradation products (MD) throughout the wound area and the constriction of laminae (LC) at the sides of the wound. The following additional notation is used: SB, subepidermal blood space; LI, limits of original incision.

*VII. Blood cells in tunic*

Four blood cell types concentrate in tunic: mature morula cells, dispersed vesicular cells, granular amoebocytes, and hyaline amoebocytes. Comparisons of blood cell concentrations in tunic, stolon tunic, and blood demonstrate that all four cell types are present in tunic at significantly higher concentrations than in blood (Table III). In tunic, all four cell types are present in greater concentration in the stolon than the body wall (Table III). Within body wall tunic, the mature morula and dispersed vesicular cells show discrete positional relationships (Figs. 4B, 7). Mature morula cells are rare in the outer areas of tunic, but are highly concentrated in tunic adjacent to the epidermis. The dispersed vesicular cells are evident in small numbers throughout the tunic and are present in extremely high concentrations at the external periphery of the tunic, particularly in inter-papillary areas. The hyaline and granular amoebocytes do not have positional sites of concentration in the tunic.

Both the mature morula and dispersed vesicular cells undergo morphological changes in the tunic. The mature morula breaks down in the tunic, either by a dispersion of its vacuoles, as if the cell membrane had been disrupted, or by a coalescence of the smaller vacuoles into one or two large vacuoles which appear to be empty. Mature morula cells which have undergone these changes in the tunic are referred to as *tunic vesicular cells*. There is no analogous break down of mature morula cells in the blood. In the tunic, the vesicles of the dispersed vesicular cell are smaller and more difficult to stain, the cytoplasm loses its affinity for stain, and the nucleus appears pycnotic.

*VII. Carbon phagocytosis*

Hyaline amoebocytes are the only cells which have engulfed carbon particles over the 48 hour period following injection into body wall tunic. Analysis of variance of mean per cent of hyaline amoebocytes with carbon as a function of

TABLE III

*The concentration of blood cells (x $10^3$) per cubic millimeter of blood and tunic with the levels of significance (P) of difference in mean concentration based on a t-test. $Tunic_B$ indicates tunic of the body wall, $tunic_S$ indicates tunic of the stolon*

| | | M.M. | D.V.C. | H.A. | G.A. |
|---|---|---|---|---|---|
| Blood | Mean | 0.54 | 0.38 | 3.14 | 2.26 |
| | S.E. | 0.10 | 0.09 | 0.58 | 0.42 |
| $Tunic_B$ | Mean | 2.84 | 11.06 | 6.43 | 6.62 |
| | S.E. | 0.66 | 1.55 | 0.89 | 0.76 |
| $Tunic_S$ | Mean | 123.18 | 47.50 | 45.32 | 47.50 |
| | S.E. | 23.99 | 9.62 | 8.96 | 9.62 |
| Blood *versus* $Tunic_B$ | *P* | 0.02 | 0.001 | 0.02 | 0.001 |
| Blood *versus* $Tunic_S$ | *P* | 0.01 | 0.01 | 0.001 | 0.01 |
| $Tunic_B$ *versus* $Tunic_S$ | *P* | 0.01 | 0.01 | 0.01 | 0.01 |

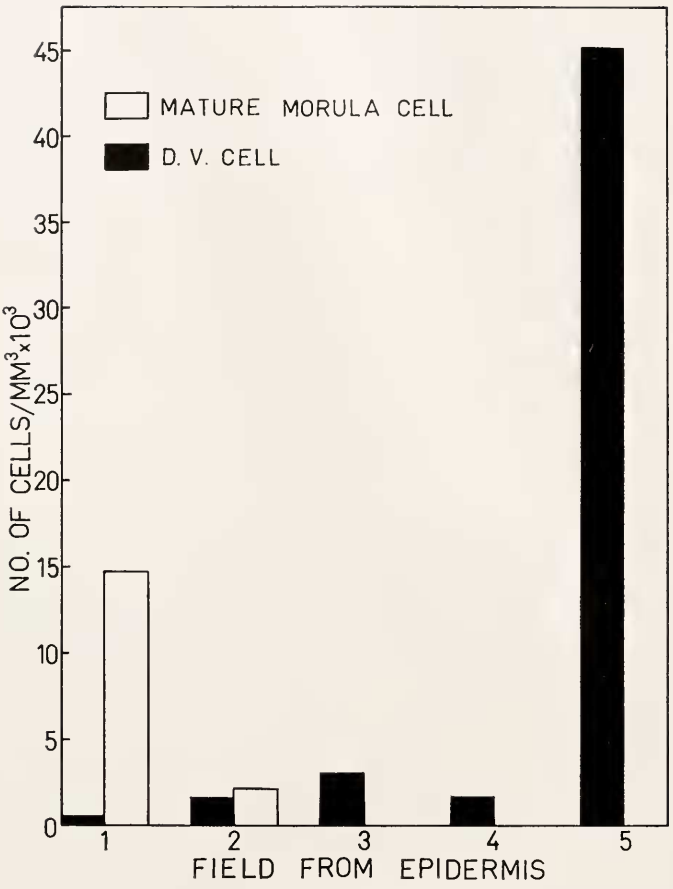


FIGURE 7. The concentration of mature morula and dispersed vesicular cells as a function of position in the tunic. Field 1 is at the epidermis and field 5 is at the external periphery of the tunic. Values reported are the mean concentrations for a sample of 5 animals with six replicate counts per animal.

time after injection gives a significant F ratio ($P < 0.01$). There is an increase in number of hyaline amoebocytes with carbon after injection with a maximum reached at 18 hours (Fig. 8). Mean comparisons of per cent of cells with carbon show that by 6 hours there are greater numbers of cells with particles than there are in controls, and this significantly higher number of cells with carbon is maintained over the 48 hour study period. The nature of the particles in the hyaline amoebocytes of the control studies is not known. However, hyaline amoebocytes often display some particulate or granular inclusions and hyaline amoebocytes which contained such particles were counted in the baseline controls to obviate misinterpretation in the phagocytosis studies.

*IX. Tunic injury*

Analyses of variance of the concentration of the four blood cell types in tunic at the site of injury as a function of day after injury reveal significant increases

in the number of mature morula cells, including the *tunic vesicular cells* ($P < 0.005$) and dispersed vesicular cells ($P < 0.005$). The maximum concentration of the two cells in the wound area is reached by the fifth day after injury (Fig. 9). By the third day after injury, there is a significantly higher concentration of mature morula cells than there is in control preparations. By the fifth day, the concentration of dispersed vesicular cells is significantly higher than controls. Fifteen days after injury, the concentration of dispersed vesicular cells remains as high as at five days after injury; however, the mature morula cell concentration has fallen. Neither the hyaline amoebocyte nor the granular amoebocyte increase significantly in the injury area over the period of study.

The injury to tunic abolishes the positional relationship of mature morula and dispersed vesicular cells. Within a day after injury, although there are higher

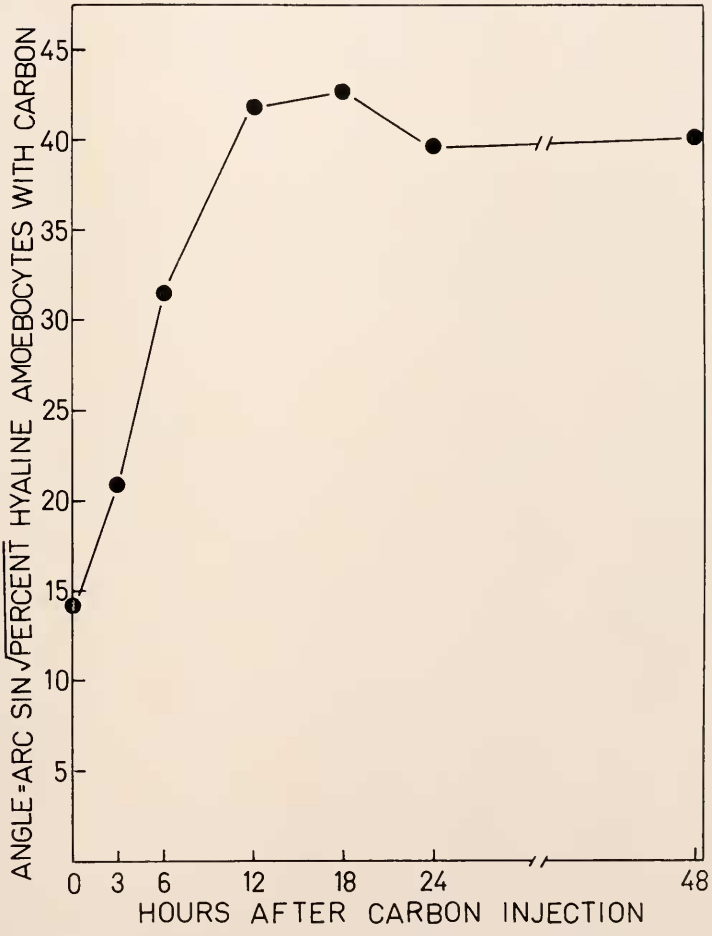


FIGURE 8. The per cent of hyaline amoebocytes with carbon particles as a function of time after carbon injection into body wall tunic. Each point represents the mean value for six animals.

levels of both cell types in the tunic, the cells are equally distributed between the inner and outer halves of the tunic injury area. By the tenth day after injury, there are significantly higher numbers of dispersed vesicular cells in the outer half of the tunic. By the fifteenth day, there is a significantly higher concentration of mature morula cells in the inner half of the injured tunic (Table IV).

As the mature morula cells and dispersed vesicular cells migrate into injured tunic, there are particular changes in both these cell types and the tunic material which they invade. The changes in the cells are essentially similar to those that take place upon migration into uninjured tunic. Initially, the mature morula cells congregate and break down at the edge of the wound. The lamination of the tunic matrix, which has been cut by the incision, is pinched together (Figs. 6C, 6D). After several days the matrix surrounding the edges of the wound is filled with a material which has staining properties similar to the mature morula

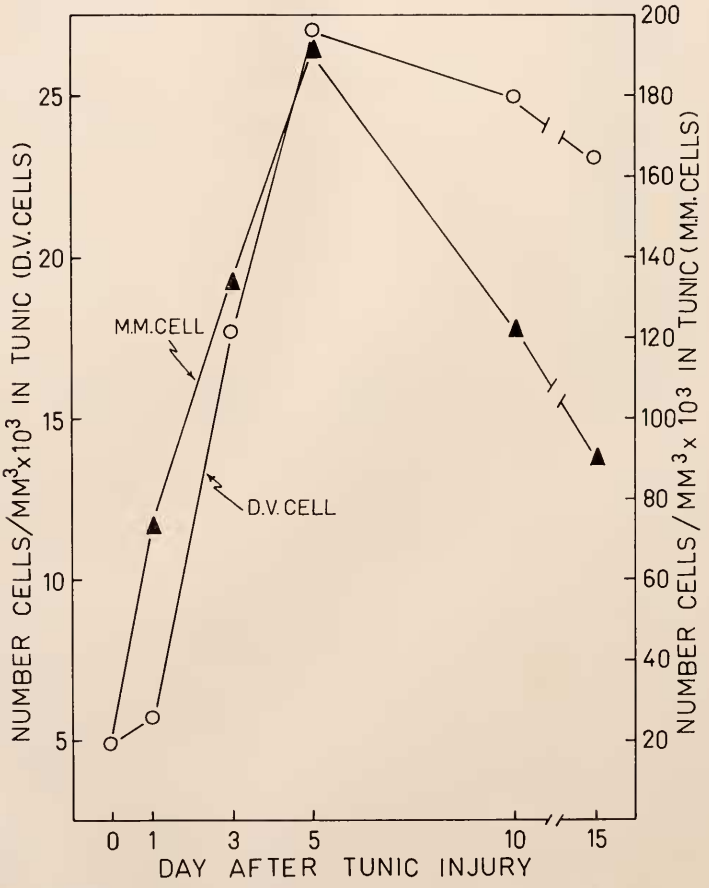


FIGURE 9. The concentration of mature morula cells, including *tunic vesicular cells,* and dispersed vesicular cells ($\times 10^3$) per $mm^3$ of tunic as a function of day after injury. The ordinate on the right refers to the mature morula cells, that on the left to the dispersed vesicular cells. Each point represents the mean value for six animals.

TABLE IV

*The significance levels of the differences in mean concentration of dispersed vesicular cells (D.V.C.) and mature morula cells (M.M.) per cubic millimeter between the inner (+) and outer (−) halves of the tunic as a function of time after injury. Also included are the significance levels of the differences in concentration of these cells in similar areas of uninjured tunic and in stolon at the half distance between the growing tip and the body wall of the animal.*

| | Cell type | |
|---|---|---|
| | M.M. | D.V.C. |
| Stolon | +0.01 | −0.001 |
| Uninjured tunic | +0.025 | −0.005 |
| Day after injury | | |
| 0 | +0.001 | N.S. |
| 1 | N.S. | N.S. |
| 3 | N.S. | N.S. |
| 5 | N.S. | N.S. |
| 10 | N.S. | −0.01 |
| 15 | +0.01 | −0.001 |

cells (Figs. 6C, 6D), and a great number of tunic vesicular cells are evident. Twenty days after injury, the external edge of the wound is covered with a dense staining cuticular material similar to the material covering the interpapillary areas of uninjured tunic. The fibrous material below the wound is lifted towards the wound and constricts there. The wound scar is denser and rougher than uninjured tunic and is not as heavily pigmented.

## DISCUSSION

It has been acknowledged generally that there is great variability in the concentration and differential distribution of blood cells in the ascidians both within and between species. There have been reports of variation in differential distribution as a function of position in the body of the tunicate, season of the year, reproductive condition, and detunication (George, 1926; Pérès, 1948a and 1948b; Millar, 1953; Cuenot cited by Andrews, 1962; Freeman, 1964). In some cases all of the blood cell types are represented in the tunic (Berrill, 1950) or only particular types are found there (Seeliger and Hartmeyer, 1911; Henze, 1913; Hecht, 1918; St. Hilaire, 1931; Pérès, 1948a, Endean, 1955b and 1961). Further, it has been stated that blood cells are located in specific areas of the tunic (Hecht, 1918; St. Hilaire, 1931; Das, 1936; Millar, 1953; Endean, 1961). Upon injury to tunic, increases in the number of blood cells in the tunic have been reported also (Hecht, 1918; St. Hilaire, 1931; Pérès, 1948b; Endean, 1961). There have been few estimations of the concentration of cells in blood or tunic (St. Hilaire, 1931; Endean, 1955a, 1955b, and 1960).

That the blood cells of the ascidians are present in areas other than the blood, such as the tunic, does not directly indicate that these areas are the sites of terminal function for the blood cells. In ascidians aggregation of blood cells in tunic could be due to a variety of factors: the tunic might be an aggregation area for senescent or dying cells or it might be one of many compartments accessible

to wandering cells whose function lies elsewhere. On the other hand, the cells might have an intermediate rather than terminal function in tunic, such as trephocytosis (Liebman, 1947) or they might have a terminal function in the tunic whether degradative or synthetic.

To gain insight into possible roles that blood cells play in the tunic and in the blood requires primarily a definite recognizable categorization of cell types and a quantitative measurement of the number and kinds of cells in the blood. Consequent to this, measurements of the partition of cells into compartments and their localization in these compartments can be verified or negated statistically, so that some certitude can be attached to the fact that particular cell types are a) present in tunic, b) are present in tunic in numbers greater than, less than, or equal to blood concentration, and c) are localized or randomly distributed within the tunic. Some of the properties of these cells in tunic then can be investigated by quantifying their response in areas of rapid tunic growth or tunic injury. By comparing the kinds and numbers of cells in rapidly growing tunic and injured tunic with blood, the selective migration of particular cell types into tunic can be verified and distinctions between growth and traumatic phenomena can be made.

Some cell functions in the tunic can be investigated directly, for example phagocytosis, while others, at this stage, must be inferred. These inferences of blood cell function must take into account the structure and function of the system in which the cells aggregate, *i.e.*, the tunic. Consequent to such preliminary studies, hypotheses follow which can be tested by a variety of means such as histochemical, biochemical, or chemical analyses of the cells and tunic.

Unfortunately there has been no statistical analysis of the number and kinds of cells in blood, in tunic, or of the partition of cells between these two systems although there has been some numerical data reported which was derived from small sample sizes.

The morphology of the blood cells from ascidians has been investigated by a number of authors (Ohuye, 1936; George, 1939; Pérès, 1943; Andrew, 1962). In general, the blood cells of ascidians can be classified into three categories: vacuolar cells, amoebocytes, and lymphocytes of stem cells. The first category includes vanadocytes, ferrocytes, and their precursors (Webb, 1939; Endean, 1955a; 1960). The second category includes the phagocytes. The third category includes those undifferentiated cells which may differentiate into the first two categories of cells (George, 1939; Endean, 1955a; 1960), or divide before differentiation into the first two categories (Pérès, 1943; 1947), or initiate budding in certain colonial species (Freeman, 1964). The signet ring, compartment cell, immature morula, and mature morula cell (which is morphologically similar to the ferrocyte and contains iron) of *H. aurantium* belong to the first category. It must be noted, however, that not all morula-like cells in ascidians are metal bearing cells; in *Perophora viridis* there are reported to be two morula-like cells. The first of these is called a morula cell but it does not stain with osmium and is non-refractile in the living state. The second morula-like cell is referred to as a green cell; it has green refractile vacuoles in the living state and stains with osmium (George, 1926; Freeman, 1964). It has been proposed that the green cell is the vanadium bearing cell in *Perophora viridis* (George, 1926). The hyaline amoebocyte, granular amoebocyte, and dispersed vesicular cell would fall

into the second category. The stem cell, the giant stem cell, and the stem cell with acidophilic vacuoles or granules belong to the third category of cells found in ascidians.

The quantitative information concerning the concentration of cells per $mm^3$ in blood or tunic is limited. In *H. aurantium,* the mean number (17,240) of cells per $mm^3$ is lower than that reported for other species of ascidian (Endean, 1955a; 1960, Freeman, 1964). The mature morula and immature morula cells of *H. aurantium,* make up approximately 30% of the circulating blood cells. This figure is lower than that reported for analogous cell types in other species (Endean, 1955a; 1960; and Freeman, 1964).

In *Pyura stolonifera,* the concentration of cells in the tunic, many of which are mature and immature morula cell analogues, has been estimated at 33,200 $mm^3$. Mature morula cell analogues are also present in the tunic of *Ascidia atra* (Hecht, 1918), *Phallusia mammillata* (Endean, 1961), and *Ascidia pygmaea* (Kalk, 1963). In *Phallusia*, deteriorated mature morula cell analogues are degraded to a bladder cell (tunic vesicular cell analogue) which are concentrated in the outer $\frac{3}{4}$'s of the tunic (Endean, 1961). The cell concentration in the tunic of *C. papillosa* is 240 per $mm^2$ (St. Hilaire, 1931). If these sections were cut at 10 microns, this would amount to 24,000 cells per $mm^3$ of tunic.

In *Halocynthia aurantium*, there are about 28,000 cells per $mm^3$ of body wall tunic. This figure is comparable with that for *Pyura* (Endean, 1955b) and *Cynthia papillosa* (St. Hilaire, 1931). The four cell types, mature morula, dispersed vesicular cell, hyaline amoebocyte, and granular amoebocyte are at significantly higher concentrations in the tunic of *H. aurantium* than they are in blood. The mature morula cell is concentrated in the basal layers of tunic adjacent to the epidermis and the dispersed vesicular cell is concentrated at the external periphery of the tunic. Both the mature morula cell and the dispersed vesicular cell undergo changes in the tunic which preclude their migration out of the tunic.

Migration of particular blood cell types into injured tunic of ascidians has been noted before (Hecht, 1918; St. Hilaire, 1931; Pérès, 1948b; Endean, 1961), but in none of the cited cases were there statistical evaluations of the injury response. Since it is difficult to standardize the degree of injury, and since there is great variation in the response of individual ascidians to injury, a careful statistical analysis of injured versus control data is necessary. Upon injury to the tunic of *H. aurantium*, only the mature morula cell and dispersed vesicular cell types are found in the injury area in significantly higher numbers than in controls. These two cell types then rapidly reassume their positional relationships in the tunic.

There is no increase in the number of hyaline amoebocytes in the injury area of tunic in *Halocynthia* over a 15 day period. The hyaline amoebocytes correspond to the macrophages of other authors (George, 1939; St. Hilaire, 1931; Endean, 1955a; 1960). In *Halocynthia*, the injection of carbon material into tunic material results in a graded increase in the number of hyaline amoebocytes with carbon particles over a 48 hour period. Since the blood was drawn from the ventral sinus rather than the site of carbon injection, the hyaline amoebocytes must be able to move in and out of the tunic. St. Hilaire (1931) has noted, that upon injection of carmine into general circulation, phagocytes would carry it into the

tunic. Since the hyaline amoebocytes of *Halocynthia* appear to be freely mobile, the failure to note an increase in number of hyaline amoebocytes in injured tunic does not rule out the possibility of increased flux.

In summary then, there is statistical evidence from this work that in *Halocynthia* certain blood cell types have a characteristic localization and concentration within the tunic which suggests that they have a secretory and/or organizational function and that one of thet cell types in tunic is a phagocyte.

The tunic of the ascidians has been categorized generally as either fibrous or gelatinous (St. Hilaire, 1931); the tunic of *H. aurantium* would be considered fibrous. Lamination of tunic fibers is of widespread, but not general, occurrence in ascidians (St. Hilaire, 1931; Millar, 1953). St. Hilaire (1931) noted, based on a sample of three animals, an increase in number of laminae with increased size of *Cynthia papillosa*. There is a definite correlation ($r = +0.95$) between the number of laminae and wet weight of *Halocynthia aurantium*.

Lamination has been explained on the basis of sea water pressure acting during tunic secretion in *Cynthia papillosa* (St. Hilaire, 1931). *H. aurantium* does not have any gas space, and the interior of the animal is, for the most part, open to the sea via the atrial cavity. At the depth at which *Halocynthia* is found, the fluctuations of pressure due to tidal changes represent a small fraction of total pressure. Thus, the bands of laminae of diverse thickness cannot be explained on the basis of pressure.

These bands of thick and thin laminae may be accounted for by seasonal variation in reserve substances available for tunic formation. *H. aurantium* has one major spawning period, in the late spring and early summer (Smith, unpubl. obs.). Fluctuations of tissue metabolites as a function of gonadal cycle and season have been reported for several marine invertebrates (Barry and Munday, 1959; Nimitz and Giese, 1964).

The lamination, itself, is probably the result of the mature morula cell's contribution to the tunic. The tunic is not secreted from the epidermis as a sheet, but as single fibers. In the region of the body wall tunic where these fibers coalesce to form laminae, the mature morula cells aggregate and break down losing their characteristic staining properties. In the tunic of the body wall and in the tunic of laboratory grown stolon proximal to the body of the ascidian, regular lamination of the fibers is evident. At the growing tips of natural and laboratory grown stolon, the tunic material is quite thin, no lamination of fibers is obvious, and the mature morula cells are positioned at the peripheral edge of the tunic material. In the wall of natural stolon, the fibers are arranged irregularly. The function of the stolon is to form a hold-fast. Since the mature morula cells are located at the exterior edge of the tunic in the growing tip of stolon, the condensation of fibers would be determined by the contours of the substrate. In natural stolon, then, it would be expected that the fibers would assume the irregular contours of the substrate. In the body wall, and in the wall of laboratory grown stolon, the mature morula cells are located just peripheral to the epidermis, and there is no directional restriction of fiber condensation as there is with natural stolon. Consequently, the coalescence of fibers in these areas, through the mediation of the mature morula cells, will assume a regularity resulting in lamination.

That the mature morula cell's function is the condensation or coalescence of

fibers in the tunic is further evidenced in injury repair studies. In this case, the initial reaction is a massive migration of mature morula cells to the external edges of the wound. In this position, the mature morula cells break down and there is a concurrent pinching together and coalescence of the edges of cut laminae. It has been suggested that mature morula cell analogues are capable of secreting tunicin in *Phallusia mammillata* and *Pyura stolonifera* (Endean, 1961; 1955b). Histochemical evidence does not support this hypothesis in the case of *Halocynthia aurantium* (Smith, 1969).

The mature morula cells decrease in relative number per unit of blood volume with increased weight of animal. They are concentrated in the body wall tunic in a restricted area adjacent to the epidermis. In this position, they degenerate which precludes the possibility of further differentiation or exit from the tunic. The decrease in relative concentration of mature morula cells may be a function of this positional relationship in the tunic. The aggregation of mature morula cells just above the epidermis resembles a surface. Since the surface and volume of a sphere or cylinder do not increase at the same rate, there could be a relative decrease in mature morula cells in respect to the volume of blood with increased size of ascidian, while a constant number of mature morula cells per unit tunic surface area is maintained.

The external surface of the ascidian tunic, particularly in the *Pyuridae*, often displays fibrous processes, spines, and cuticle structures (Berrill, 1950), and *Halocynthia* has both cuticle and spines. Kennedy (1966) has shown that a tunic cell in *Ascidia aspersa*, which may be analogous to the dispersed vesicular cell, will take up radioiodine and this cell is incorporated into the cuticle. It has been proposed that the cuticle and spines of *Cynthia papillosa* are composed of the same substance as the tunic matrix, but that the mode of deposition differs (St. Hilaire, 1931). St. Hilaire, (1931) suggested further that the dispersed vesicular cell analogue in *Cynthia* does not make a substantial contribution to the substance of the spines and cuticle. This does not appear to be true, however, for *Halocynthia.*

In natural stolons, laboratory grown stolons, body wall tunic, and injured tunic two properties stand out; a high concentration of dispersed vesicular cells at the periphery of the tunic material, and the presence of spines or cuticle in these areas. The spines are spaced at regular intervals over the surface of body wall tunic, irrespective of size of animal. Therefore, as the ascidian grows, there must be an absolute increase in the number of spines. The most obvious region for the initiation of the growth of new spines is from the interpapillary surface between older spines. These regions are particularly rich in dispersed vesicular cells.

Both the cuticle and spines have analogous staining properties which differ from those of the tunic matrix (Smith, 1969). Further, the blood dispersed vesicular cells have some histochemical properties in common with spine material and the vesicles of the dispersed vesicular cell stain for protein (Smith, 1969). The dispersed vesicular cells at the edge of tunic material display characteristics which could indicate that they are expended functionally: vesicles are small and more difficult to stain, the cytoplasm has lost its ability to take up stains, and the nucleus appears necrotic.

It is proposed that the dispersed vesicular cells have the function of forming cuticle and spines. Spines will be elaborated upon completion of the initial growth process which may involve mature morula cells. In natural stolon, there is a surface-to-substrate contact which may inhibit the elaboration of spines. In injury repair studies, initially, the edge of the wound mimics the tips of growing stolons in that there is a cuticle present and both mature morula and dispersed vesicular cells are present and not positionally separated. Given sufficient time, the edge of the wound might elaborate spines. An analogous situation is found in laboratory grown stolon. At the growing tip no spines are present, but there is a graded development of spines along the length of stolon with fully developed spines at the juncture of the body wall and stolon. In all cases, including the surface of interpapillary areas of body wall tunic, a cuticle is secreted and dispersed vesicular cells are present.

The dispersed vesicular cells also decrease in relative concentration in blood with increased weight of animal and accumulate in tunic material as a surface aggregation. The arguments presented to explain the decrease in relative number of mature morula cells in the blood also are valid for the dispersed vesicular cells (see above).

The relative number of hyaline amoebocytes in blood increase with increased weight of ascidian. There are no obvious areas or organs in *H. aurantium* which display large aggregations of deteriorating hyaline amoebocytes, such as is found for the dispersed vesicular cells and mature morula cells in the tunic. The increase in relative numbers of hyaline amoebocytes in blood cannot be explained as a reciprocal function of the decrease in other cell types because there is no difference in the absolute concentration of blood cells per unit blood volume as a function of weight. However, if the circulating life span of hyaline amoebocytes exceeds that of the mature morula cells, dispersed vesicular cells, and their precursors, there might be an accumulation of hyaline amoebocytes with time as reflected in increased weight of animal.

The granular amoebocyte is an enigma. It does not possess staining characteristics which are common to any of the tunic structures (Smith, 1969). It does not change in relative number with change in weight of ascidian. This may be accepted as an argument that its function is relative to a given volume of blood rather than a target organ or area. It is possible that it is a coagulocyte, or has a lysosomal function but there is no definite evidence for this.

The stem cell in *H. aurantium* agrees morphologically with the lymphocyte category of other authors. It is accepted generally that the lymphocyte type gives rise to other blood cell types in ascidians (George, 1939; Pérès, 1943; Endean, 1955a; 1960). This investigation does not offer evidence which would negate this hypothesis.

It has been suggested that in *Phallusia mammillata* (Endean, 1960) and *Pyura stolonifera* (Endean, 1955a), the histogenetic pathway for the development of the vanadocyte and ferrocyte, which are analogous to the mature and immature morula cells of *H. aurantium*, is from the lymphocyte (stem), to the signet ring cell, to the compartment cell, to the vanadocyte or ferrocyte. In *H. aurantium*, the signet ring cells, compartment cells, immature morula cells, and mature morula cells stain for the presence of iron as do the ferrocyte and its vacuolated precursors in *Pyura stolonifera* (Endean, 1955a). This study does not offer evidence which would contradict Endean's scheme (1955a; 1960) for metal-bearing blood cell differentiation.

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## Summary

1. Then morphological blood cell types are recognized in *Halocynthia aurantium*. The mature morula cells, dispersed vesicular cells, stem cells, and stem cells with acidophilic vacuoles or granules decrease in differential distribution with increased weight of animal. The hyaline amoebocyte increases in differential distribution with increased weight of animal.

2. There is no significant difference in total blood cell concentration per cubic millimeter of blood as a function of weight of animal.

3. The mature morula cells, dispersed vesicular cells, hyaline amoebocytes, and granular amoebocytes are at significantly higher concentrations in the tunic of the body wall and stolon than they are in the blood. The mature morula cell is concentrated in the tunic just peripheral to the epidermis and the dispersed vesicular cell is concentrated at the external limits of the tunic. The hyaline amoebocyte is a phagocyte.

4. Upon injury to the tunic, the mature morula cells and dispersed vesicular cells increase significantly in the wound area over a 15 day period. The positional relationships of these cells are rapidly reinstated in the tunic.

5. The tunic consists of three components: epidermis, fibrous matrix, and cuticle with spines. The matrix consists of fibrous laminae which increase in number with increased weight of animal.

6. The mature morula cells, immature morula cells, compartment cells, and signet ring cells contain protein bound iron.

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